(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 March 2004 (04.03.2004)

PCT

(10) International Publication Number
WO 2004/018501 A1

(51) International Patent Classification[7]: C07K 1/04

(21) International Application Number: PCT/KR2002/001604

(22) International Filing Date: 26 August 2002 (26.08.2002)

(25) Filing Language: English

(26) Publication Language: English

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/018501 A1

(54) Title: METHOD FOR SYNTHESIZING PEPTIDES

(57) Abstract: The present invention relates to a novel method for synthesizing peptides in solution, the method comprising: a) treating a peptide or amino acid derivative, which has an α-amine function blocked with a urethane group of the formula R^1-SO_2-CH_2CH_2-O-CO-, wherein R^1 is aryl, with an excess of an aliphatic secondary amine in an organic solvent to provide the release of the free α-amine function of said amino acid or peptide derivative and the formation of a tertiary amine adduct between said secondary amine and the liberated vinyl compound of the formula R^1-SO_2-CH_2=CH_2; b) removing the solvent and the excess of said secondary amine; c) contacting thus formed mixture of the N_α-deprotected amino acid or peptide derivative and said tertiary amine adduct with subsequent peptide or amino acid derivative N_α-protected with the above indicated or another urethane group under conditions providing the formation of a peptide bond between the free α-amine function of the N_α-deprotected amino acid or peptide derivative and an α-carboxylic function of the subsequent N_α-protected peptide or amino acid derivative; d) separating the newly formed peptide from the reaction mixture; e) repeating procedures as set forth above until the desired polypeptide is obtained.

METHOD FOR SYNTHESIZING PEPTIDES

Technical Field

The present invention relates to a novel method for synthesizing peptides in solution, which provides substantial facilitation and acceleration of the whole synthetic procedure.

Background Art

A basic problem in peptide synthesis is one of blocking or protecting the α-amino group from interaction with a carboxyl group on the same amino acid. These undesirable side reactions are prevented by attaching to one amino acid a group that will render the -NH_2 group unreactive and still permit the desired reaction to proceed. In addition to providing protection for the amino group, the blocking group is preferably one that can be easily removed without chemically altering the remainder of the molecule including the peptide linkage that has been built up during the synthesis. The peptide chain assembly usually consists of multiple consecutive synthetic cycles, and each cycle includes two basic chemical stages: 1) removal of a protecting group from the α-amino group of a peptide being built (N_α-deprotection stage), and 2) coupling of the N_α-deprotected peptide with subsequent N_α-protected amino acid or peptide segment. Therefore, the ease and swiftness of performing the N_α-deprotection stage stipulate for the rate and effectiveness of the overall process of the peptide assembly.

The vast majority of amino protecting groups reported for peptide syntheses have centered around three groups: carbobenzoxy (Z or Cbz), cleaved by catalytic hydrogenation; tert-butoxycarbonyl (Boc or t-Boc), cleaved by mild acidolysis; and 9-fluorenylmethoxycarbonyl (Fmoc), cleaved by secondary amines (for a review see, e.g. Kocienski, P. *Protecting Groups*, Corrected Edition, Thieme: Stuttgart-N.-Y., **2000**; Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis,* 3rd Ed.; John Wiley & Sons, Inc.: New York, NY, **1999**). These groups are orthogonal to each other because the Boc group is stable to secondary amines and hydrogenolysis, the Fmoc group is stable

to acidolysis, and the Z group is stable to secondary amines and mild acidolysis. Each of these protecting groups has found its place in peptide chemistry.

Hydrogenolytic cleavage of the Z group from short peptides is simple and clean; the work-up comprises just removing the catalyst and evaporating the solvent. However, with longer peptides the reaction may appear very slow. In addition, the presence of sulfur-containing amino acids in a peptide renders impossible catalytic hydrogenation.

Cleavage of the Boc group produces only volatile by-products, so the work-up represents removing an acidic cleavage reagent. Complete removal of the acid, which is critical for successful proceeding of the following coupling stage, requires in many cases multiple evaporations, careful washing and drying of an N_{α}-deprotected peptide.

The Fmoc group, a base-labile protecting group widely utilized in contemporary peptide synthesis, has revealed numerous advantages: it has excellent acid stability; it is readily cleaved in a non-hydrolytic fashion by a variety of amines *via* base-promoted β-elimination; other standard peptide protecting groups (i.e. Boc, benzyl) can be removed in its presence; and, the amine generated upon cleavage is the free base (Atherton, E.; Sheppard, R.C., in *The Peptides*; Udenfriend, S.; Meienhofer, J., Eds.; Academic Press: New York, NY, **1987**; Vol. 9, p. 1.). However, while the Fmoc methodology has found broad application in automated solid phase peptide synthesis, it has several shortcomings which hamper its use in solution peptide synthesis. First, Fmoc-protected amino acids (and peptides) have poor stability in both weakly basic and neutral DMF solutions usually employed at the coupling stage (Atherton, E.; Bury, C.; Sheppard, R.C.; Williams, B.J. *Tetrahedron Lett.* **1979**, 3041; Bodanszky, M.; Deshmane, S.S.; Martinez, J. *J. Org. Chem.* **1979**, *44*, 1622.). Second, and perhaps most detrimental, is a relatively slow and reversible reaction of the Fmoc cleavage alkene product, dibenzofulvene, with an excess of the cleaving reagent, secondary aliphatic amine. It makes difficult efficient trapping of dibenzofulvene with the amine and its subsequent removal from a reaction mixture, and eventually leads to the contamination of the target peptide product with the sparingly soluble dibenzofulvene polymer.

It is apparent that there is a need in efficient and flexible methods for solution peptide synthesis which could share the advantages of the Fmoc N_α-protection while being devoid of its drawbacks.

Disclosure of Invention

Some of the authors of the present invention have found earlier that urethane amino protecting groups derived from 2-arylsulfonylethanols can be cleaved by amines under non-hydrolytic conditions *via* base-promoted β-elimination, a mechanism very similar to that described for the Fmoc cleavage (Samukov, V.V.; Sabirov, A.N.; Troshkov, M.L. *Zh. Obshchei Khim.* **1988**, *58*, 1432). But surprisingly, in contrast to the Fmoc, the urethane cleavage product, aryl vinyl sulfone, is trapped very rapidly and irreversibly when an excess of a secondary aliphatic amine is employed as a cleavage reagent (Samukov, V.V.; Sabirov, A.N.; Pozdnyakov, P.I. *Tetrahedron Lett.* **1994**, *35*, 7821; Sabirov, A.N.; Kim, Y.-D.; Kim, H.-J.; Samukov, V.V. *Protein Peptide Lett.* **1997**, *4*, 307). In this case the product of trapping represents a vinyl sulfone-amine adduct, i.e. tertiary amine, so the removal of the excess of the cleavage reagent and the solvent gives an equimolar mixture of an N_α-deprotected peptide and the above tertiary amine. It has been then assumed that there is no need to separate this mixture before commencing the coupling stage, because tertiary amines are always added to the coupling mixture in order to maintain the basicity of the medium and to promote the acylation reaction, so already present molar equivalent of the amine may well serve for these purposes.

The present invention therefore relates to a novel method for synthesizing peptides in solution, which makes use of the findings described above and provides substantial facilitation and acceleration of the whole synthetic procedure.

Accordingly, an object of the invention is the method for synthesizing peptides comprising:

a) treating a peptide or amino acid derivative, which has an α-amine function blocked with a urethane group of the formula

$\mathbf{R^1\text{-}SO_2\text{-}CH_2CH_2\text{-}O\text{-}CO\text{-}}$,

wherein $\mathbf{R^1}$ is aryl,

with an excess of an aliphatic secondary amine in an organic solvent to provide the release of the free α-amine function of said amino acid or peptide derivative and the formation of a tertiary amine adduct between said secondary amine and the liberated vinyl compound of the formula

$\mathbf{R^1\text{-}SO_2\text{-}CH_2{=}CH_2}$;

b) removing the solvent and the excess of said secondary amine;

c) contacting thus formed mixture of the N_α-deprotected amino acid or peptide derivative and said tertiary amine adduct with subsequent peptide or amino acid derivative N_α-protected with the above indicated or another urethane group under conditions providing the formation of a peptide bond between the free α-amine function of the N_α-deprotected amino acid or peptide derivative and an α-carboxylic function of the subsequent N_α-protected peptide or amino acid derivative;

d) separating the newly formed peptide from the reaction mixture;

e) repeating procedures as set forth above until the desired polypeptide is obtained.

The radical $\mathbf{R^1}$ in the urethane structure may be substituted or unsubstituted aryl, however, in order to provide a reasonably high rate of urethane cleavage by base-promoted β-elimination it is preferable to employ aryls bearing strong electron-withdrawing substituents. Among such substituted aryl radicals the most preferable are 4-nitrophenyl and 4-sulfonylated phenyls. At $\mathbf{R^1}$ = 4-nitrophenyl the N_α-protecting group represents known 2-(4-nitrophenyl)sulfonylethoxycarbonyl (Nsc) group (Samukov, V.V.; Sabirov, A.N.; Pozdnyakov, P.I. *Tetrahedron Lett.* **1994**, *35*, 7821; U.S. Patents 5,616,788; 6,265,590). The group of 4-sulfonylated phenyls can be exemplified by the 2-(4-methylsufonylphenyl)sulfonylethoxycarbonyl (Mpc) group already known in the art as an amino protecting group (Verhart, C.; Tesser, G. *Rec. Trav. Chim. Pays-Bas.* **1988**, 107, 621). Other preferred embodiments of the group include, for example, 4-phenylsulfonylphenyl and a variety of 4-sulfonamide derivatives, namely, 4-dimethylamidosulfonylphenyl; 4-diethylamidosulfonylphenyl; 4-morpholidosulfonyl-phenyl; 4-piperididosulfonylphenyl.

Cleavage rates of the above described urethanes by the amine-promoted β-elimination are lower than that of the Fmoc group under comparable conditions, but, on the

other hand, these amino protecting groups are markedly more stable than the Fmoc in weakly basic or neutral reaction solutions usually employed at the coupling stage.

In order to provide the rapid cleavage of the above described N_α-protecting groups and the efficient trapping of liberated vinyl sulfone compounds it is desirable for a secondary amine employed as a cleavage and scavenging reagent to combine properties of a strong base and a potent nucleophile. Also important are physical properties of the amine. The separation of an excess of the amine from a reaction mixture after the N_α-deprotection reaction may be achieved in a variety of ways, however, the most simple and expeditious way is the vacuum distillation (evaporation). It is thus desirable for the amine to be volatile (i.e., to have a low boiling point). Among amines which meet all these requirements the most preferable are: dimethylamine; diethylamine; di-n-propylamine; piperidine, pyrrolidine; morpholine. The excess of an amine required for efficient cleavage and trapping may vary from 2 to 100 molar equivalents; the optimal excess for a particular case can easily be selected by a practitioner in the area of peptide chemistry.

Solvents suitable for conducting the N_α-deprotection stage are preferably polar, aprotic and volatile solvents capable of dissolving starting materials and reaction products, such as dichloromethane, acetonitrile, tetrahydrofuran, dioxane, N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone, dimethylsulfoxide, and the like.

Thus formed volatile cleavage reagent can easily be removed by evaporation after performing the N_α-deprotection. Usually a single additional co-evaporation with a fresh portion of a solvent is sufficient to secure the complete removal of a secondary amine from a reaction mixture.

After removal of an excess of an amine and a solvent, e.g. by evaporation, the equimolar mixture of an N_α-deprotected amino acid or peptide derivative and a vinyl sulfone-amine adduct is further introduced without any additional work-up into the coupling reaction with the next peptide or amino acid derivative N_α-protected with the above indicated or another urethane group. The activation of an α-carboxylic function of the N_α-protected component for coupling can be achieved by a variety methods known in the art, for example, by the preceding formation of active esters, such as 4-nitrophenyl, pentachlorophenyl, pentafluorophenyl, 1-hydroxybenzotriazolyl esters or other known

types of active esters; by the conversion into symmetric or mixed anhydrides, or into azides. Coupling may also be performed in the presence of known coupling reagents, e.g. dicyclohexylcarbodiimide, diisopropylcarbodiimide, benzotriazolyl-1-oxy(tris-dimethylamino)phosphonium hexafluorophosphate (BOP), numerous uronium type reagents. When adjusting the basicity of a reaction medium during the coupling reaction, one should take into account that one molar equivalent of base is already present in the reaction mixture.

Isolation of the newly formed peptide from the reaction mixture can be performed by usual procedures known in the art, e.g. extraction, precipitation, washing, chromatography, etc.

The whole sequence of procedures described above may be repeated until the desired polypeptide chain is constructed.

Where desired or as required, side chains of amino acid or peptide derivatives can be protected with appropriate protecting groups during synthetic cycles and then deprotected to obtain the final product. The nature and type of protecting groups that can be used, their application, and their subsequent removal are well documented in the literature and known to those skilled in the art.

Best Mode for Carrying Out the Invention

The invention will now be described by way of examples which are provided as illustration and are not intended as being limiting. All of the amino acids in the following description have L-configuration unless otherwise indicated. Abbreviations for functional and protective groups, solvents, reagents, etc., as used herein are listed below:

Boc – tert-butoxycarbonyl

BOP - benzotriazolyl-1-oxy(tris-dimethylamino)phosphonium hexafluorophosphate

Cbz – benzyloxycarbonyl

DCC – N,N'-dicyclohexylcarbodiimide

Despsc - 2-(4-diethylamidosulfonyl)phenylsulfonylethoxycarbonyl

DMF – N,N-dimethylformamide

Dmspsc - 2-(4-dimethylamidosulfonyl)phenylsulfonylethoxycarbonyl

FC – flash-chromatography

Fmoc – 9-fluorenylmethoxycarbonyl

HOBt – 1-hydroxybenzotriazole

Mpc - 2-(4-methylsufonyl)phenylsulfonylethoxycarbonyl

Mspsc - 2-(4-morpholidosulfonyl)phenylsulfonylethoxycarbonyl

NMM – N-methylmorpholine

Nsc - 2-(4-nitrophenylsulfonyl)ethoxycarbonyl

Pfp - pentafluorophenyl

Pipspsc - 2-(4-piperididosulfonyl)phenylsulfonylethoxycarbonyl

Pspsc - 2-(4-phenylsulfonyl)phenylsulfonylethoxycarbonyl

tBu – tert-butyl

THF – tetrahydrofuran

TFA – trifluoroacetic acid

TEA – triethylamine

Chromatographic mobility values R_f are shown for thin-layer chromatography sheets Alufolien Kieselgel 60 F_{254} (Merck, Darmstadt, Germany); chloroform/methanol/acetic acid, 95:5:3 (A) and 90:10:3 (B), and ethyl acetate/pyridine/acetic acid/water, 60:5:15:10 (C), have been used as developing solvents, spots are detected by UV-absorbance and/or by ninhydrin reaction. Molecular ion masses $(M+H)^+$ have been measured using MALDI-TOF VISION 2000 device (Thermo Bioanalysis, England).

Example 1: 2-(4-Phenylsulfonyl)phenylsulfonylethyl chloroformate (Pspsc-Cl)

a. 2-(4-Phenylsulfonyl)phenylsulfonylethanol

To a stirred solution of 2-(4-chlorophenyl)sulfonylethanol (4.41 g) and thiophenol (3.1 g) in 10 ml of DMF, 1.4 g of KOH in 5 ml of ethanol is added. Stirring is continued for 10 h at ambient temperature, then the mixture is diluted with 70 ml of water and extracted with 100 ml of ethyl acetate. The extract is washed with 5% aq. $NaHCO_3$ and brine, dried over anhydrous sodium sulfate and evaporated at reduced pressure. The oily residue is then dissolved in 30 ml of acetone. To the solution, 3 ml of 0.3 M aq. sodium

molybdate is added, then, with stirring, 8 ml of 30% hydrogen peroxide. The mixture is kept for 6 h at 50°C, evaporated and treated with 100 ml of water. The precipitate is filtered off, washed with water, dried and recrystallized from ethyl acetate-petroleum to give 3.6 g of the title product; mp 107-108°C; R_f 0.35 (A). For $C_{14}H_{14}O_5S_2$ (FW 326.39) calcd.: C 51.52%, H 4.32%; found: C 51.14%, H 4.40%.

b. Pspsc-Cl.

To a cooled at –10°C and stirred mixture of THF (35 ml) and 30% toluene solution of phosgene (10 ml), finely powdered 2-(4-phenylsulfonyl)phenylsulfonylethanol (3.2 g) is added, and the stirring is continued overnight at ambient temperature. The mixture is evaporated to dryness, the solid residue was treated with toluene, separated by filtration, washed with toluene and petroleum and dried in the vacuum desiccator. Yield 3.55 g; mp 160-161°C. For $C_{15}H_{13}ClO_6S_2$ (FW 388.85) calcd.: C 46.33%, H 3.37%; found: C 45.94%, H 3.48%.

Example 2: 2-(4-Dimethylamidosulfonyl)phenylsulfonylethyl chloroformate (Dmspsc-Cl)

a. N,N-Dimethyl-4-bromobenzenesulfonamide

To a stirred and cooled (ice bath) mixture of 16 ml of 30% aq. dimethylamine and 50 ml of dioxane, 4-bromobenzenesulfonyl chloride (7.7 g) is added portion-wise during 10 min. The mixture is evaporated and treated with plenty of water, the precipitate is filtered off, washed with cold water and directly used at the next stage.

b. 2-(4-Dimethylamidosulfonyl)phenylsulfonylethanol

A mixture of crude N,N-dimethyl-4-bromobenzenesulfonamide, 3 ml of 2-mercaptoethanol and 30 ml of DMF is supplemented with 18 ml of 2 n. ethanolic solution of KOH and warmed for 8 h at 70°C. The mixture is diluted with 200 ml of water and extracted with 2 x 70 ml of ethyl acetate. The extract is washed with 5% aq. $NaHCO_3$ and brine, dried over anhydrous sodium sulfate and evaporated at reduced pressure. The oily residue is then dissolved in 50 ml of acetone. To the solution, 4 ml of 0.3 M aq. sodium molybdate is added, then, with stirring, 10 ml of 30% hydrogen peroxide. The mixture is kept for 5 h at 50°C, evaporated and treated with 100 ml of water. The precipitate is

filtered off, washed with water, dried, then washed with ethyl acetate and petroleum. Yield 7.1 g of the title product; mp 171-173°C; R_f 0.27 (A). For $C_{10}H_{15}NO_5S_2$ (FW 293.36) calcd.: C 40.94%, H 5.15%, N 4.77%; found: C 41.35%, H 5.05%, N 4.42%.

c. Dmspsc-Cl

Prepared from 2-(4-dimethylamidosulfonyl)phenylsulfonylethanol as described in the Example 1b. Mp 135°C (softening), 144°C. For $C_{11}H_{14}ClNO_6S_2$ (FW 355.82) calcd.: C 37.13%, H 3.97%, N 3.94%; found: C 37.42%, H 4.05%, N 3.77%.

Following compounds are also prepared by the above method:

2-(4-Piperididosulfonyl)phenylsulfonylethanol; mp 181-182°C; R_f 0.34 (A). For $C_{13}H_{19}NO_5S_2$ (FW 333.43) calcd.: C 46.83%, H 5.74%, N 4.20%; found: C 47.18%, H 5.65%, N 4.05%.

2-(4-Piperididosulfonyl)phenylsulfonylethyl chloroformate (Pipspsc-Cl); mp 185-187°C. For $C_{14}H_{18}ClNO_6S_2$ (FW 395.88) calcd.: C 42.48%, H 4.58%, N 3.54%; found: C 42.32%, H 4.65%, N 3.42%.

2-(4-Morpholidosulfonyl)phenylsulfonylethanol; mp 190-191°C; R_f 0.23 (A). For $C_{12}H_{17}NO_6S_2$ (FW 335.40) calcd.: C 42.97%, H 5.11%, N 4.18%; found: C 42.68%, H 5.25%, N 4.01%.

2-(4-Morpholidosulfonyl)phenylsulfonylethyl chloroformate (Mspsc-Cl); mp 166-169°C. For $C_{13}H_{16}ClNO_7S_2$ (FW 397.86) calcd.: C 39.25%, H 4.05%, N 3.52%; found: C 39.61%, H 4.15%, N 3.39%.

2-(4-Diethylamidosulfonyl)phenylsulfonylethanol; mp 167-168°C; R_f 0.30 (A). For $C_{12}H_{19}NO_5S_2$ (FW 321.42) calcd.: C 44.84%, H 5.96%, N 4.36%; found: C 44.48%, H 6.08%, N 4.21%.

2-(4-Diethylamidosulfonyl)phenylsulfonylethyl chloroformate (Despsc-Cl); mp 155-157°C. For $C_{13}H_{18}ClNO_6S_2$ (FW 383.87) calcd.: C 40.68%, H 4.73%, N 3.65%; found: C 40.21%, H 4.85%, N 3.49%.

Example 3: N_α-Protected amino acids

To a suspension of an amino acid (3.6 mmol) in 10 ml of anhydrous chloroform, chlorotrimethylsilane (8 mmol) is added, then, with stirring and cooling, TEA (8 mmol).

After stirring for 1 h at 40°C, to a resultant clear solution a chloroformate (3 mmol, Examples 1, 2) is added, and the stirring is continued for 3 h at ambient temperature. The mixture is then evaporated, the residue is distributed between cold 0.5 n. aq. HCl (20 ml) and ethyl acetate (30 ml). The organic phase is washed with 0.5 n. aq. HCl and water, dried over anhydrous sodium sulfate and evaporated at reduced pressure. The residue is recrystallized from an appropriate solvent or subjected to flesh-chromatography on a silica gel column. N_α-Protected amino acids prepared by this method are listed in the Table 1.

Nsc- and Mpc-amino acids are prepared according to published procedures.

Table 1: N_α-Protected amino acids

Entry No.	N_α-Protected amino acid	Yield, %	Mp, °C	$[\alpha]_D^{20}$ (c 1, DMF)	Purification method
1	Pspsc-Phe-OH	70	160-162	- 16.3°	acetone-ether
2	Dmspsc-Phe-OH	55	130-132	- 15.9°	FC
3	Pipspsc-Phe-OH	72	143-145	- 15.9°	FC
4	Mspsc-Phe-OH	52	166-168	- 14.6°	FC
5	Pspsc-Val-OH	70	178-179	- 7.8°	FC
6	Dmspsc-Val-OH	65	142-144	- 7.5°	FC
7	Pipspsc-Val-OH	75	162-164	- 6.7°	
8	Mspsc-Val-OH	72	187-189	- 7.9°	FC
9	Despsc-Val-OH	71	155-157	- 7.4°	FC
10	Pspsc-Gly-OH	80	170-171		acetone-ether
11	Dmspsc-Gly-OH	77	156-159		FC
12	Pipspsc-Gly-OH	75	195-197		ethanol-ether
13	Mspsc-Gly-OH	62	200-203		FC
14	Despsc-Gly-OH	72	182-184		FC
15	Pspsc-Leu-OH	75	141-143	- 16.9°	FC
16	Dmspsc-Leu-OH	62	158-160	- 16.2°	FC
17	Pipspsc-Leu-OH	78	176-178	- 14.9°	FC
18	Mspsc-Leu-OH	70	162-163	- 16.0°	FC
19	Dmspsc-Asp(OtBu)-OH	80	128-129	- 10.8°	FC
20	Mspsc-Glu(OtBu)-OH	80	168-169	- 8.4°	FC
21	Pipspsc-Pro-OH	75	180-181	- 19.7°	FC

Example 4: Synthesis of Tyr-Gly-Gly-Phe-Leu (Leucine-enkephalin)

a. Mspsc-Phe-Leu-OtBu

Leucine tert-butyl ester hydrochloride (0.6 mmol), Mspsc-Phe-OH (0.5 mmol, entry 4, Table 1) and HOBt (0.74 mmol) are dissolved in 2 ml DMF. To the solution, NMM (2 mmol) and BOP (0.6 mmol) are added, and the mixture is kept for 60 min at ambient

temperature. The mixture is then distributed between 25 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with ether to yield 83% of the title dipeptide; R_f 0.50 (A).

b. Pipspsc-Gly-Phe-Leu-OtBu

The dipeptide (Example 4a) is dissolved in DMF-piperidine mixture (1:4, v/v; 3 ml) and, after 10 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 3 ml of DMF and dissolved in DMF (2 ml). To the solution, Pipspsc-Gly-OH (0.5 mmol, entry **12**, Table 1), HOBt (0.74 mmol), NMM (1 mmol) and BOP (0.6 mmol) are added, and the mixture is kept for 1.5 h at ambient temperature. The mixture is then distributed between 25 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with ether to yield 86% of the title tripeptide; R_f 0.32 (A).

c. Dmspsc-Gly-Gly-Phe-Leu-OtBu

The tripeptide (Example 4b) is dissolved in DMF-piperidine mixture (1:4, v/v; 3 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 3 ml of DMF and dissolved in DMF (2 ml). To the solution, Dmspsc-Gly-OH (0.45 mmol, entry **11**, Table 1), HOBt (0.6 mmol), NMM (0.8 mmol) and BOP (0.5 mmol) are added, and the mixture is kept for 1.5 h at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with ether-petroleum to yield 93% of the title tetrapeptide; R_f 0.55 (B).

d. Boc-Tyr(Boc)-Gly-Gly-Phe-Leu-OtBu

The tetrapeptide (Example 4c) is dissolved in DMF-piperidine mixture (1:4, v/v; 3 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 3 ml of DMF and dissolved in DMF (3 ml). To the solution, Boc-Tyr(Boc)-OPfp (0.4 mmol) is added, and the mixture is kept for 30 min at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic

phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with petroleum ether to yield 90% of the title pentapeptide; R_f 0.75 (B).

e. Tyr-Gly-Gly-Phe-Leu

The protected pentapeptide (Example 4d) is dissolved in the mixture chloroform-TFA-water (50:50:2, 10 ml) and kept for 1 h at ambient temperature. The solution is then evaporated to dryness, the residue is triturated with ether. Reprecipitation from ethanol with ether gives 160 mg of leucine-enkephalin trifluoroacetate (48% as calculated on starting Mspsc-Phe-OH); R_f 0.27 (C); m/z = 556.5, $M+H^+$ (calcd. 556.77); purity 94% by HPLC.

The above synthesis has been repeated using various combinations of N_α-protective groups and N_α-deblocking reagents. Thus performed synthetic runs are summarized in the Table 2. N- and C-terminal amino acids, scale of synthesis and coupling conditions are as described above. Amino acid residues are enumerated from N- to C-terminus; total yields and purity are given as above for crude deprotected leucine-enkephalin trifluoroacetate.

Table 2: Syntheses of leucine-enkephalin

N_α-Protection (Entry No.)			N_α-Deblocking reagent (reaction time, min)	Total yield, %	Purity, %
Gly-2	Gly-3	Phe-4			
Nsc	Nsc	Nsc	Piperidine/DMF, 1:4 v/v (10)	52	95
Nsc	Nsc	Nsc	Diethylamine/DMF, 1:4 v/v (15)	46	92
Nsc	Nsc	Nsc	Morpholine/DMF, 1:2 v/v (30)	44	93
Nsc	Nsc	Nsc	Dimethylamine/DMF, 2 M (20)	39	93
Nsc	Nsc	Nsc	Piperidine/MeCN, 1:4 v/v (15)	42	92
Nsc	Nsc	Nsc	Dipropylamine/DMF, 1:3 v/v (15)	37	93
Mpc	Mpc	Mpc	Piperidine/DMF, 1:4 v/v (15)	50	92
Pspsc (10)	Despsc (14)	Pspsc (1)	Piperidine/dimethylacetamide, 1:4 v/v (15)	44	91
Dmspsc (11)	Dmpsc (11)	Dmpsc (2)	Pyrrolidine/DMF, 1:4 v/v (15)	49	92

Example 5: Synthesis of Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu (THF-γ2)

a. Nsc-Lys(Boc)-Phe-Leu-OtBu

Mspsc-Phe-Leu-OtBu (0.87 mmol, Example 4a) is N_α-deprotected as described in the Example 4b and dissolved in 4 ml of DMF. To the solution, Nsc-Lys(Boc)-OH (1

mmol), HOBt (1.5 mmol), BOP (1.1 mmol), and NMM (2 mmol) are added, and the mixture is kept for 1 h at ambient temperature. The mixture is then distributed between 25 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated and left for the crystallization of the product. The precipitate is filtered off and washed with ether to yield 86% of the title tripeptide; R_f 0.72 (B).

b. Nsc-Pro-Lys(Boc)-Phe-Leu-OtBu

The tripeptide (Example 5a) is dissolved in DMF-piperidine mixture (1:4, v/v; 5 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 3 ml of DMF and dissolved in DMF (5 ml). To the solution, Nsc-Pro-OH (0.8 mmol), HOBt (1.2 mmol), NMM (1.8 mmol) and BOP (0.85 mmol) are added, and the mixture is kept for 1.5 h at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with ether to yield 91% of the title tetrapeptide; R_f 0.60 (B).

c. Nsc-Gly-Pro-Lys(Boc)-Phe-Leu-OtBu

The tetrapeptide (Example 5b) is dissolved in DMF-piperidine mixture (1:4, v/v; 5 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 5 ml of DMF and dissolved in DMF (5 ml). To the solution, Nsc-Gly-OH (0.8 mmol), HOBt (1.2 mmol), NMM (1.8 mmol) and BOP (0.85 mmol) are added, and the mixture is kept for 2.5 h at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with water, 0.5 n. aq. HCl, again with water, and evaporated to dryness. The residue is treated with ethyl acetate-ether to yield 94% of the title pentapeptide; R_f 0.50 (B).

d. Mspsc-Glu(OtBu)-Asp(OtBu)-OH

To a cooled solution of Mspsc-Glu(OtBu)-OH (1 mmol) and pentafluorophenol (1.2 mmol) in 5 ml of THF, DCC (1 mmol) is added, and the mixture is stirred for 2 h at ambient temperature. The precipitate is filtered off, the filtrate is evaporated and combined with 1.1 mmol of H-Asp(OtBu)-OH in 4 ml of DMF. The mixture is stirred for 12 h until clear solution is formed, then distributed between 30 ml of ethyl acetate and 30 ml of cold 0.25 n. aq. HCl. The organic phase is separated, washed subsequently with cold 0.25 n. aq.

HCl and water, and evaporated to dryness. The residue is treated with ether to yield 88% of the desired dipeptide; R_f 0.25 (A).

e. Boc-Leu-Glu(OtBu)-Asp(OtBu)-OH

The dipeptide (Example 5d) is dissolved in DMF-piperidine mixture (1:4, v/v; 5 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 2 x 5 ml of DMF and dissolved in DMF (5 ml). To the solution, Boc-Leu 4-nitrophenyl ester (1.1 mmol) and HOBt (0.2 mmol) are added. The mixture is kept for 3 h at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 30 ml of cold 0.25 n. aq. HCl. The organic phase is separated, washed subsequently with cold 0.25 n. aq. HCl and water, and evaporated to an oil. The residue is washed by tritutation with petroleum (2 x 10 ml), then dried under vacuum to yield 74% of the target tripeptide as a solid foam; R_f 0.40 (A).

f. Boc-Leu-Glu(OtBu)-Asp(OtBu)-Gly-Pro-Lys(Boc)-Phe-Leu-OtBu

The pentapeptide (0.38 mmol, Example 5c) is dissolved in DMF-piperidine mixture (1:4, v/v; 3 ml) and, after 15 min, evaporated to dryness at 1 mm Hg. The residue is co-evaporated with 5 ml of DMF and dissolved in DMF (3 ml). To the solution, Boc-Leu-Glu(OtBu)-Asp(OtBu)-OH (0.41 mmol, Example 5e), HOBt (1 mmol), NMM (1 mmol) and BOP (0.55 mmol) are added, and the mixture is kept for 4 h at ambient temperature. The mixture is then distributed between 35 ml of ethyl acetate and 20 ml of 5% aq. $NaHCO_3$, the organic phase is separated, washed subsequently with 5% aq. $NaHCO_3$, 0.5 n. aq. HCl, and water, and evaporated to oil. The residue is treated with ether-hexane to yield 90% of the title protected octapeptide; R_f 0.45 (A).

g. Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu

The protected octapeptide (440 mg) is dissolved in 10 ml of cold TFA-water mixture (9:1, v/v) and stirred for 40 min at ambient temperature. The solution is evaporated, the thick oily residue is treated with cold ether. The precipitate is filtered off, washed with ether and dried under vacuum to yield 380 mg of the crude octapeptide (87% purity by HPLC). The peptide is dissolved in 4% aq. acetic acid and purified by preparative reversed phase chromatography on a Lichroprep RP18 column using water-ethanol gradient

buffered with acetic acid. Final yield is 280 mg of 98% pure octapeptide; m/z = 919.5, $M+H^+$ (calcd. 919.13); $[\alpha]_D^{20}$ = - 68.9° (c 1,.1% aq. CH_3COOH).

Industrial Applicability

The present invention provides a novel method for synthesizing peptides in solution, thereby substantially facilitating and accelerating the whole synthetic procedure.

Claims

1. A method for synthesizing peptides comprising:

a) treating a peptide or amino acid derivative, which has an α-amine function blocked with a urethane group of the formula

R^1-SO_2-CH_2CH_2-O-CO-,

wherein R^1 is aryl,

with an excess of an aliphatic secondary amine in an organic solvent to provide the release of the free α-amine function of said amino acid or peptide derivative and the formation of a tertiary amine adduct between said secondary amine and the liberated vinyl compound of the formula

R^1-SO_2-CH_2=CH_2;

b) removing the solvent and the excess of said secondary amine;

c) contacting thus formed mixture of the N_α-deprotected amino acid or peptide derivative and said tertiary amine adduct with subsequent peptide or amino acid derivative N_α-protected with the above indicated or another urethane group under conditions providing the formation of a peptide bond between the free α-amine function of the N_α-deprotected amino acid or peptide derivative and an α-carboxylic function of the subsequent N_α-protected peptide or amino acid derivative;

d) separating the newly formed peptide from the reaction mixture;

e) repeating procedures as set forth above until the desired polypeptide is obtained.

2. The method of claim 1 wherein R^1 is selected from the group consisting of 4-nitrophenyl, 4-phenylsulfonylphenyl, 4-methylsulfonylphenyl, 4-dimethylamidosulfonyl-phenyl, 4-diethylamidosulfonylphenyl, 4-morpholidosulfonylphenyl and 4-piperidido-sulfonylphenyl.

3. The method of claim 1 wherein the aliphatic secondary amine is selected from the group consisting of dimethylamine, diethylamine, di-n-propylamine, piperidine, pyrrolidine and morpholine.

4. The method of claim 1 wherein the organic solvent is a volatile aprotic solvent.

5. The method of claim 4 wherein the volatile aprotic solvent is selected from the group consisting of dichloromethane, acetonitrile, tetrahydrofuran, dioxane, N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone and dimethylsulfoxide.

6. The method according to claims 3 or 4 wherein removing the solvent and the excess of the aliphatic secondary amine is performed by evaporation.

7. The method of claim 1 wherein said contacting step is performed in the presence of a coupling reagent.

8. The method of claim 7 wherein said coupling reagent is selected from the group consisting of dicyclohexylcarbodiimide, diisopropylcarbodiimide, benzotriazolyl-1-oxy(tris-dimethylamino)phosphonium hexafluorophosphate (BOP) and uronium type coupling reagents.